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| <p>(54) Title: NOVEL ANTIPLATELET AGENT</p> <p>(57) Abstract</p> <p>This invention combines the unique antiplatelet effects of S-nitrosothiols and the antiadhesive properties of fragments of vWF in the A1 domain to provide unique molecules that exploit both of these properties. Preferred molecules comprise a fragment of A1 (ala444-asn730) in which arginine at position 545 is replaced by cysteine (the most frequent von Willebrand disease type 2b mutation) that has been discovered to impair platelet adhesion, and to exhibit antithrombotic activity <i>in vivo</i>. This cysteine residue may be S-nitrosated to produce a novel molecule that has the potential for impairing platelet adhesion as well as activation/aggregation, and such molecules form the basis of a novel therapeutic method for impairing platelet responses following vascular injury or in other thrombotic disorders according to this invention.</p> <p>COPIES FETES A LA Biblioteca de<br/>Patents CIDEM / UB-FBG<br/>AMB UNA IMPRESSORA LASER<br/><b>RICOH</b></p> |    |  |

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## NOVEL ANTIPLATELET AGENT

### BACKGROUND OF THE INVENTION

#### Field of the Invention

This invention is directed to anti-thrombotic therapy using novel anti-thrombotic agents. In particular, the anti-thrombotic agents are S-nitrosated polypeptides which bind to platelet receptor glycoprotein GPIb/IX.

#### 5 Review of Related Art

##### *von Willebrand Factor and Platelet Function*

During the past several years significant progress has been made in understanding the molecular aspects of platelet function with regard to both their role in normal hemostasis and the development of pathological vascular occlusion. The first event in normal primary hemostasis or  
10 development of arterial thrombosis is the binding (adhesion) of platelets to the subendothelium at sites of vascular injury. This first step occurs by binding of von Willebrand factor (vWF) to the platelet receptor glycoprotein Ib/IX (GPIb/IX) following its binding to components of exposed subendothelium. Thus, vWF acts as a "bridging" molecule between platelets and the vessel wall. As a consequence of vWF binding to GPIb, glycoprotein IIb/IIIa (GPIIb/IIIa) is activated through  
15 a complex signaling pathway leading to platelet aggregation mediated by fibrinogen or, under conditions of high shear stress, by vWF itself (Ruggeri, et al., 1982, *Proc. Natl. Acad. Sci. USA*, 79:6038-6041; Ruggeri, et al., 1983, *J. Clin. Invest.*, 72:1-12; Ikeda, et al., 1991, *J. Clin. Invest.*, 87:1234-1240). Furthermore, the binding of vWF to GPIb can mediate events that are associated with the effects of agonists like ADP and thrombin, such as activation of GPIIb/IIIa and support of  
20 platelet aggregation. This indicates that vWF serves as more than the "glue" that mediates platelet

- 2 -

adhesion to the vessel wall but also induces and modulates other later steps of hemostasis and thrombogenesis.

vWF is a polymeric glycoprotein that circulates in plasma as a series of multimers with molecular weights ranging from  $0.25 \times 10^6$  daltons to  $20 \times 10^6$  daltons. In addition to its role in platelet adhesion, it carries and stabilizes factor VIII in the circulation (Sadler, 1991, *J Biol. Chem.*, 266:22777-22780). The vWF gene, located on chromosome 12, spans 178 kb and is interrupted by 51 introns (Ginsburg, et al., 1985, *Science*, 228:1401; Mancuso, et al., 1989, *J. Biol. Chem.*, 264:19514-19527.) vWF is synthesized from an 8.7 kb mRNA and is expressed in endothelial cells and megakaryocytes. Synthesis of vWF is a complex multistep process that results in the generation of a precursor protein, pre-pro-vWF (Meyer, et al., 1993, *Thromb. Haemost.*, 70:99-104).

This large molecule comprises a 22 amino acid (aa) signal peptide, as well as provWF, which consists of a 741 aa propeptide and a 2050 aa mature subunit. These 250 kDa subunits assemble into multimers of up to 100 subunits (Wagner, 1990, *Annu. Rev. Cell Biol.*, 6:217-246). After dimerization by disulfide bonding at carboxyterminal domains in the endoplasmic reticulum, further multimerization takes place in the Golgi or post-Golgi compartments through disulfide linkages at amino-terminal domains.

In the blood vessel, vWF is constitutively secreted by endothelial cells. vWF is also stored within intracellular granules in both endothelial cells (Weibel-Palade bodies) and platelets ( $\alpha$ -granules). These specialized granules release vWF in response to a variety of stimuli including vascular damage. The vWF stored within these granules contains larger multimers than those which are constitutively secreted by endothelial cells. These high-molecular-weight (HMW) multimers are more effective in platelet binding than smaller sized multimers (Gralnick, et al., 1981, *Blood*,

- 3 -

58:397-397; Federici, et al., 1989, *British Journal of Hematology*, 73:93-99); therefore, rapid release of stored vWF into the circulation may be particularly useful in the setting of vessel injury.

The provWF consists of four types of repeated domains (A to D) and has two disulfide loops: one is located between cys 509 and 695 in the A1 domain and the other between cys 923 and 1109 in the A3 domain (Meyer, et al., 1993, *Thromb. Haemost.*, 70:99-104). Progress has been made in identifying specific regions of the vWF subunit that are important for function. The A1 domain contains binding sites for GPIb, sulfatides, and heparin. Using proteolytic or recombinant fragments of vWF, the binding domain for GPIb has been located within the T116 fragments (aa 449-728) which overlaps the A1 loop (Fujimura, et al., 1986, *J. Biol. Chem.*, 261:381-385; Cruz, et al., 1993, *J. Biol. Chem.*, 268:21238-21245; Sugimoto, et al., 1991, *Biochemistry*, 30:5202-5209; Gralnick, et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89:7880-4; Azuma, et al., 1991, *J. Biol. Chem.*, 266:12342-12347; Pietu, et al., 1989, *Biochem. Biophys. Res. Commun.*, 164:1339-1347; Andrews, et al., 1989, *Biochemistry*, 28:8326-8336).

Under its native conformation, human vWF does not spontaneously interact with GPIb. The exposure of the GPI-binding site of vWF can be regulated by a series of physiological or non-physiological events. Most of these events appear to modify the structure and/or the conformation of the A1 region. The binding of vWF to the subendothelium, which spontaneously occurs via the T116 sequence (Denis, et al., 1993, *Arterioscler. Thromb.*, 13:398-406), is responsible for the subsequent exposure of the GPIb-binding site of vWF (Sakariassen, et al., 1979, *Nature*, 279:635-638). Similarly, collagen and heparin bind to vWF via sequences in the A1 loop (Mohri, et al., 1989, *J. Biol. Chem.*, 264:17361-17367) and modulate its interaction with GPIb. Binding of vWF to collagen promotes its interaction with GPIb while binding to heparin inhibits this interaction

- 4 -

(Fressinaud, et al., 1988, *J. Lab. Clin. Med.*, 112(1):58-67; Sobel, et al., 1991, *J. Clin. Invest.*, 87:1787-1793; Savage, et al., 1992, *J. Biol. Chem.*, 267(16):11300-11306).

The interaction of vWF with non-physiologic modulators of its binding to GPIb also involves sequences close to or within the A1 loop. The immobilization of vWF on a plastic surface, for example, leads to platelet adhesion via GPIb (Berndt, et al., 1992, *Biochemistry*, 31:11144-11151). The interaction of vWF with ristocetin involves 474-488 and 692-708 sequences flanking the A1 loop, whereas botrocetin binds to four sequences within this loop (514-542, 539-553, 569-583 and 629-643) (Sugimoto, et al., 1991, *J. Biol. Chem.*, 266:18172-18178; Ginsburg, et al., 1993, *Thromb. Haemost.*, 69:177-184). The inhibition of vWF binding to GPIb by polyanionic compounds like aurin tricarboxylic acid (ATA) involves positively charged sequences of the A1 loop (Girma, et al., *Thromb. Haemost.*, 68:707-13, 1992). Finally, the GPIb-binding site can be achieved by the removal of the sialic acid residues from the carbohydrate side chains of vWF (Gralnick, et al., 1985, *J. Clin. Invest.*, 75:19-25). Since 9 of the 22 carbohydrate chains of vWF are within the T116 fragment but outside the A1 loop, the net local decrease of the negative charges may be responsible for the exposure of the GPIb-binding site.

Studies of patients with von Willebrand disease (vWD) have confirmed the role of the conformation of the A1 domain in the regulation of vWF binding to GPIb. vWF from patients with type 2B vWD is characterized by an increased capacity to bind to platelet GPIb. Mutations of this type have been identified within the 505-698 aa residues (Ginsburg, et al., *Thromb. Haemost.*, 69:177-84, 1993). The expression of recombinant, mutated vWF has confirmed the direct role of these mutations in the increased affinity of vWF for GPIb (Randi, et al., 1992, *J. Biol. Chem.*, 267:21187-21192; Inbal, et al., 1993, *Thromb. Haemost.*, 70:1058-1062; Cooney, et al., 1992, *Proc.*

- 5 -

*Natl. Acad. Sci. USA*, 89:2869-2872; Kroner, et al., 1992, *Blood*, 79:2048-2055).

Matsushita, et al. (1995, *J. Biol. Chem.*, 270:13406-13414) recently performed charged-to-alanine mutagenesis of the vWF A1 domain to examine the roles of specific charged residues in the interaction of vWF with platelet GPIb. By this approach, amino acid residues Glu596 and Lys599 appeared to be important in the interaction of vWF with platelet GPIb. Furthermore, alanine substitutions at Arg545, the site of the type 2b mutations studied by Inbal, et al. (1993, *Thromb. Haemost.*, 70:1058-62), and the segments between Glu496-Arg511 and Arg687-Glu689 resulted in spontaneous binding of vWF to GPIb. The striking distribution of positive and negative charges in distinct regions of the A1 domain suggests that intramolecular electrostatic interactions among these sites play a major role in the regulation of vWF binding to GPIb. An acidic segment, Asp252 and Asp287, of the GPIb a chain was identified as a binding site for vWF (Murata, et al., 1991, *J. Biol. Chem.*, 266:15474-15480).

#### *Nitric Oxide and Platelet Function*

An important determinant of the anti-thrombotic properties of the normal endothelium is its ability to inhibit platelet activation, adhesion, and aggregation. Two principal endothelial products account for these antiplatelet effects: prostacyclin and endothelium-derived relaxing factor (EDRF)/NO (Loscalzo, et al., 1996, in "Methods in Nitric Oxide Research," M. Feelisch and J. S. Stamler, editors, John Wiley & Sons, Ltd., Chichester, U.K., pp. 584-591). These substances act synergistically to inhibit platelet function, and do so through cAMP- and cGMP-dependent mechanisms, respectively (Loscalzo, et al., 1996). Importantly, while both prostacyclin and NO inhibit platelet activation and aggregation, only NO is effective as an inhibitor of platelet adhesion

- 6 -

(deGraaf, et al., 1992, *Circ.*, 85:2284-2290). The importance of EDRF-mediated inhibition of platelet function *in vivo* is best illustrated by the observation that inhibition of NO synthesis can be accompanied by intravascular thrombosis (Shultz, et al., 1992, *J. Clin. Invest.*, 90:1718-1725, Freedman, et al., 1996, *J. Clin. Invest.*, 97:979-987). Inhibition of platelet function by NO can be  
5 potentiated by thiols (Loscalzo, et al., 1996), and this effect is probably a consequence of the formation of thionitrites or S-nitrosothiols (Loscalzo, et al., 1996). These NO adducts are potent platelet inhibitors that form *in vivo* and are probably responsible for stabilizing NO and for many of the biological effects attributed to NO directly in the vasculature (Loscalzo, et al., 1996). Previous work has shown that S-nitrosation of a protein avid for subendothelium (*i.e.*, serum albumin) impairs  
10 platelet adhesion and smooth muscle proliferation following vascular injury (Marks, et al., 1995, *J. Clin. Invest.*, 96:2630-2638).

#### SUMMARY OF THE INVENTION

It is an object of this invention to provide new antithrombotic compositions which impair  
15 platelet activation and adhesion.

It is another object of this invention to provide a compound which competes with endogenous von Willebrand's Factor for binding to GPIb/IX but does not activate platelets upon binding to GPIb/IX.

It is another object of this invention to provide a method of treating an atherothrombotic  
20 disorder administering to patients new antithrombotic compositions which impair platelet activation and adhesion. These and other objects are met by one or more of the following embodiments.

In one embodiment, this invention provides a polypeptide which binds to platelet receptor



- 7 -

glycoprotein Ib/IX (GPIb/IX) and competes with mature von Willebrand's Factor for binding to GPIb/IX, but does not activate platelets upon binding to GPIb/IX. The peptide of this invention contains at least one cysteine residue which is S-nitrosated. Preferably, the polypeptide according to this invention contains at least a portion of the sequence of the A1 domain of von Willebrand's factor. More preferably, the polypeptide according to this invention is polythiolated and a plurality of thiol groups are nitrosated.

In another embodiment, this invention provides a substantially pure polypeptide containing at least the amino acid sequence of von Willebrand's factor from ala at position 444 to asp at position 730, but where the amino acid residue at position 545 is cys. Preferably, the polypeptide according to this embodiment has at least one cysteine residue which is S-nitrosated. More preferably, the polypeptide according to this embodiment is polythiolated and a plurality of thiol groups are nitrosated.

In yet another embodiment, this invention provides a method of treating a patient having an atherothrombotic disorder by administering to the patient a pharmaceutical composition containing a polypeptide which binds to platelet receptor glycoprotein Ib/IX (GPIb/IX) and competes with endogenous von Willebrand's Factor for binding to GPIb/IX, but does not activate platelets upon binding to GPIb/IX. Preferably the polypeptide used in this method contains at least one cysteine residue which is S-nitrosated. More preferably, the polypeptide according to this invention contains at least a portion of the sequence of the A1 domain of von Willebrand's factor. Even more preferably, the polypeptide according to this invention is polythiolated and a plurality of thiol groups are nitrosated.

The inventors have combined the unique antiplatelet effects of S-nitrosothiols and the

- 8 -

antiadhesive properties of fragments of vWF in the A1 domain, thereby providing unique molecules that exploit both of these properties. One preferred molecule comprises a fragment of A1 (ala444-asp730) in which arginine at position 545 is replaced by cysteine (the most frequent von Willebrand disease type 2b mutation) that has been shown by the inventors to impair platelet adhesion, and to exhibit antithrombotic activity *in vivo*. This cysteine residue is S-nitrosated, to produce a molecule that is denoted S-NO-AR545CvWF. This unique molecule has the potential for impairing platelet adhesion as well as activation/aggregation, and as such forms the basis of a novel therapeutic strategy for impairing platelet responses following vascular injury or in other thrombotic disorders.

## DETAILED DESCRIPTION OF THE EMBODIMENTS

### *Antiplatelet Therapy*

In both thrombosis and reclusion of coronary arteries, platelet adhesion is the initiating event. Thrombolytic therapy (streptokinase, tissue-type plasminogen activator) benefits patients who have suffered a myocardial infarction by reducing mortality and infarct size (Loscalzo, et al., 1996: Gruppo Italiano per lo Studio della Streptomachinasi nell'Infarto Miocardico, 1986, *Lancet*, 1:397-402; ISIS-2 (Second International Study of Infarct Survial) Collaborative Group, 1988, *J. Am. Coll. Cardiol.*, 12:3A-13A). However, the benefit is limited by incomplete reperfusion, a delayed recanalization time, and occurrence of thrombotic reclusion in up to 15% of cases (Van de Werf, et al., 1990, *British Med. J.*, 297:1374-1379; Colleen, 1990, *Ann. Intern. Med.*, 112:529-538; Colleen, et al., 1991, *Prig. Cardiovasc. Dis.*, 34:101-102). Importantly, thrombolytic therapy is also accompanied by increased platelet activation by the direct action of plasmin on the platelet, by the elaboration of thrombin through the action of plasmin in generating prothrombinase, and by the

- 9 -

exposure of subendothelial collagen following lysis of the occlusive thrombus (Loscalzo, et al., 1995, *Thromb. Haemost.*, 74:291-293).

Since the first event in thrombogenesis is the recognition of vessel wall-bound vWF by platelets through the GPIb receptor, it is apparent that the selective inhibition of binding of endogenous vWF to GPIb as adjunctive therapy for thrombolysis would be an appropriate early intervention, likely to result in a beneficial anti-thrombotic effect. It was previously shown that the tryptic fragment of vWF of 52/48 kDa comprising residues Val449 and Lys728 inhibits the binding of native vWF to GPIb (Fujimura, et al., 1986, *J. Biol. Chem.*, 261:381-385). Moreover, the GPIb-binding domain of vWF expressed in *E. coli* has been shown to inhibit the binding of native vWF to GPIb (Sugimoto, et al., 1991, *Biochemistry*, 30:5202-5209), and unlike native vWF bound to GPIb, does so in the absence of any modulator. These previous studies provided the rationale for the use of a recombinant vWF fragment as an antiplatelet/anti-thrombotic agent. Indeed, recently, several fragments of vWF expressed in *E. coli* containing the A1 domain have been shown to bind to the platelet membrane GPIb receptor and inhibit the interaction of vWF with platelets (Gralnick, et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89:7880-7884); Mohri, et al., 1993, *Peptides*, 14:125-129).

Experimental studies with VCL (Bio-Technology General, Inc.), another recombinant fragment of von Willebrand factor that spans from leu504 to lys728, showed delayed thrombus formation and reclusion in dogs (Yao, et al., 1994, *Circulation*, 89:2822-2828). This fragment was also shown to have local anti-thrombotic effects on nitrogen laser-induced thrombus formation in guinea pig mesenteric arteries without compromising general hemostasis and, importantly, without prolonging bleeding time (Azzam, et al., 1995, *Thromb. Haemost.*, 73:318-323).

The inventors have discovered that recombinant mutant vWF fragment AR545C can bind

- 10 -

to the platelet surface receptor involved in adhesion and that this mutant fragment exhibits anti-thrombotic activity *in vitro*. The inventors have also discovered that nitric oxide congeners, such as S-nitroso-N-acetyl-L-cysteine and S-nitroso-serum albumin, have antiplatelet effects. Nitric oxide delivered by these S-nitrosothiols inhibits platelet function by a variety of interrelated mechanisms and, importantly, is a powerful inhibitor of platelet adhesion, a property not shared by the other potent endothelial product, prostacyclin.

Nitrosated (S-NO-AR545C),<sup>3</sup> or polynitrosated mutant vWF fragment, (pS-NO-AR545C), provides targeted delivery of nitric oxide to the platelet through its S-nitrosothiol functional group(s). The targeted delivery of NO, based on AR545C binding to platelet glycoprotein Ib, will impair platelet activation and aggregation directly, an effect not manifest by the vWF fragments themselves, and thereby impairs platelet adhesion by two distinct mechanisms. These mechanisms at the very least are additive, but may also be synergistic, increasing the potency of the pharmacologic effects significantly.

The novel compounds of this invention represent a potential novel class of therapeutic agents useful for the treatment of atherothrombotic disorders. AR545C manifests its anti-thrombotic properties without prolonging the bleeding time in experimental animals. With the polynitrosated derivative, the toxic/therapeutic ratio (therapeutic index) for this class of antiplatelet agents will be significantly improved over existing agents.

Compounds of this invention are generally characterized in that they compete with von Willebrand factor for binding to platelet membrane protein GP Ib/IX. Generally, the compounds of this invention will not activate platelets upon binding to GP Ib. Additionally, compounds according to this invention contain one or more nitrosated thiol groups. Usually, the base compound prior to

- 11 -

nitrosation will not activate platelets; however, so long as the nitrosated compound fails to activate platelets, the compound may be within the contemplation of this invention. Particular preferred compounds are polypeptides derived from the amino acid sequence of the A1 domain of von Willebrand's factor. Fragments of von Willebrand's factor which compete for binding to receptor protein GP Ib are described above, and these fragments are suitable starting points for the compounds of this invention. Where such peptide fragments are capable of binding GPIb but do not contain cysteine residues, thiol groups for nitrosation may be added by homocysteine thiolactone derivatization of epsilon- or alpha-amino groups through ester links by the methods of Benesch and Benesch (1958, *Proc. Natl. Acad. Sci. USA*, 44:848-853). Alternatively, analogs of the fragments may be prepared using recombinant DNA techniques by expression of a nucleic acid sequence corresponding to a portion of the von Willebrand sequence except for point mutation of one or more codons to encode cysteine residues in the expressed polypeptide.

The practice of the present invention employs, unless otherwise indicated, conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are well known to the skilled worker and are explained fully in the literature. *See, e.g.,* Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins, eds., 1985); "Transcription and Translation" (B.D. Hames & S.J. Higgins, eds., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1986); "Immobilized Cells and Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989).

- 12 -

The sequence of vWF is readily available to those skilled in the art, for instance in Genbank under accession No. X04385. DNA segments or oligonucleotides having specific sequences can be synthesized chemically or isolated by one of several approaches. The basic strategies for identifying, amplifying and isolating desired DNA sequences as well as assembling them into larger DNA molecules containing the desired sequence domains in the desired order, are well known to those of ordinary skill in the art. *See, e.g.,* Sambrook, et al., (1989); B. Perbal, (1984). Preferably, DNA segments corresponding to all or a portion of the vWF sequence may be isolated individually using the polymerase chain reaction (M.A. Innis, et al., "PCR Protocols: A Guide To Methods and Applications," Academic Press, 1990). A complete sequence may be assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. *See, e.g.,* Edge (1981), *Nature*, 292:756; Nambair, et al. (1984), *Science*, 223:1299; Jay, et al. (1984), *J. Biol. Chem.*, 259:6311.

The assembled sequence can be cloned into any suitable vector or replicon and maintained there in a composition which is substantially free of vectors that do not contain the assembled sequence. This provides a reservoir of the assembled sequence, and segments or the entire sequence can be extracted from the reservoir by excising from DNA in the reservoir material with restriction enzymes or by PCR amplification. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice (*see, e.g.,* Sambrook, et al., incorporated herein by reference). The construction of vectors containing desired DNA segments linked by appropriate DNA sequences is accomplished by techniques similar to those used to construct the segments. These vectors may be constructed to contain additional DNA segments, such as bacterial origins of replication to make shuttle vectors (for shuttling between prokaryotic

- 13 -

hosts and mammalian hosts), etc.

Procedures for construction and expression of mutant proteins of defined sequence are well known in the art. A DNA sequence encoding a mutant form of vWF or a fragment thereof can be synthesized chemically or prepared from the wild-type sequence by one of several approaches, including primer extension, linker insertion and PCR (*see, e.g.,* Sambrook, et al.). Mutants can be prepared by these techniques having additions, deletions and substitutions in the wild-type sequence. It is preferable to test the mutants to confirm that they are the desired sequence by sequence analysis and/or the assays described below. Mutant vWF fragments for testing may be prepared by placing the coding sequence for the polypeptide in a vector under the control of a promoter, so that the DNA sequence is transcribed into RNA and translated into protein in a host cell transformed by this (expression) vector. The mutant fragments may be produced by growing host cells transfected by an expression vector containing the coding sequence for the mutant under conditions whereby the polypeptide is expressed. The selection of the appropriate growth conditions is within the skill of the art.

#### **Producing the Recombinant Peptide**

Preferably, DNA from the selected clones should be subcloned into an expression vector, and the polypeptide expressed by cells transformed with the vector may be tested for immunoreactivity with antibodies against vWF or the polypeptide of this invention. Such subcloning is easily within the skill of the ordinary worker in the art in view of the present disclosure. The amino acid coding region of the DNA sequence for this invention may be longer or shorter than the coding region of vWF so long as the recombinant peptide expressed by the DNA sequence retains ability to bind GPIIb. The preparation of selected clones which contain DNA sequences corresponding to all or part

- 14 -

of the sequence of vWF may be accomplished by those of ordinary skill in the art using conventional molecular biology techniques along with the information provided in this specification and the vWF sequence as disclosed herein.

Where clones are selected from an expression library, selection may be accomplished by expressing the library sequences and detecting the expressed peptides immunologically. Clones are selected that express peptides which bind antibodies specific for vWF or which bind GPIIb. These selection procedures are well known to those of ordinary skill in the art (*see, e.g.,* Sambrook, et al.). Hybridization using a nucleic acid whose sequence corresponds to the sequence of vWF may be used to select clones corresponding to a portion of the vWF gene.

#### Cloning for Expression

Once a coding sequence for the desired polypeptide sequence has been prepared or isolated, it can be cloned into any suitable vector or replicon and thereby maintained in a composition which is substantially free of vectors that do not contain the coding sequence (*e.g.,* free of other clones from the library). Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice (*see, e.g.,* Sambrook, et al., incorporated herein by reference). The DNA sequences and DNA molecules of the present invention may be expressed using a wide variety of host/vector combinations. According to the present invention, the coding sequence is placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence is transcribed into RNA in the host cell transformed by a vector containing this expression construct. The coding sequence may or may not contain a signal peptide or leader sequence.

Of course, not all host/expression vector combinations function with equal efficiency in



- 15 -

expressing the DNA sequences of this invention or in producing the polypeptides of this invention. However, a particular selection of a host/expression vector combination may be made by those skilled in the art. For example, the selection should be based on a balancing of a number of factors. These include compatibility of the host and vector, toxicity of the proteins encoded by the DNA  
5 sequence to the host, ease of recovery of the desired protein, expression characteristics of the DNA sequences and the expression control sequences operatively linked to them, biosafety, costs and the folding, form or any other necessary post-expression modifications of the desired protein. Preferably, the host cell will not express proteases which degrade the recombinant polypeptide of this invention.

10 Depending on the expression system and host selected, the protein is produced by growing host cells transformed by an expression vector containing the coding sequence for a polypeptide cross-reactive with the hpr gene product under conditions whereby the protein is expressed. The protein is then isolated from the host cells and purified. The selection of the appropriate growth conditions and recovery methods are within the skill of the art. Suitable expression vector and host  
15 cell systems are well known to those of ordinary skill in the art, and are taught, for instance, in Sambrook, et al., 1989. The peptide may be obtained by growing the transformed cells in culture under conditions wherein the cloned DNA is expressed. Depending on the expression vector chosen, the peptide may be expressed as a fusion protein or a mature protein which is secreted or retained intracellularly, or as an inclusion protein. The desired polypeptides can be recovered from the  
20 culture by well-known procedures, such as centrifugation, filtration, extraction, and the like, with or without cell rupture, depending on how the peptide was expressed. The crude aqueous solution or suspension may be enriched for the desired peptide by protein purification techniques well known

- 16 -

to those skilled in the art.

Purified polypeptides prepared as described herein may be readily nitrosated by S-nitroso-glutathione using standard procedures of direct nitrosation or trans-S-nitrosation (Zhang, et al., 1996, *Meth. Neurosci.*, 31:41-46; Zhang, et al., 1996, *J. Biol. Chem.*, 271:14271-14279). Other procedures  
5 for nitrosating thiol groups on polypeptides which are known to those skilled in the art may also be used.

The skilled worker will of course confirm that molecules prepared according to this invention have the required properties of competitive binding to the GPIIb platelet receptor in a manner which competes with vWF and does not activate platelets. Suitable assays are set forth in detail in the  
10 examples below, including inhibition of ristocetin-induced platelet aggregation, inhibition of platelet aggregation induced by ADP-thrombin, and inhibition of platelet adhesion in a flow model. Further confirmatory assays may include demonstration of clot lysis in a rabbit femoral thrombosis model (see Rudd, et al., 1992, *Circ. Res.*, 70:829-834) or radiolabeled platelet accretion model (see Marks, et al., 1995, *J. Clin. Invest.*, 96:2630-2638).

5 Anti-thrombotic molecules according to this invention provide a new mode of therapy for atherothrombotic arterial diseases and venous thrombotic diseases. Atherothrombotic arterial diseases within the contemplation of this invention include coronary artery disease, such as stable angina pectoris, acute coronary syndromes, such as unstable angina pectoris and acute myocardial infarction; cerebral vascular disease, including acute stroke and transient ischemic attack; mesenteric  
10 arterial disease; mesenteric ischemia "abdominal angina," and mesenteric infarction; as well as peripheral arterial disease, including acute peripheral arterial occlusion and intermittent claudication. Compounds of this invention are also useful during treatment of coronary artery disease, including

- 17 -

anti-thrombotic therapy during coronary angioplasty, anti-thrombotic therapy during cardiopulmonary bypass, and limiting of platelet activation during ischemia reperfusion. Venous thrombotic diseases which may be treated according to this invention include deep venous thrombosis and pulmonary thromboembolism. These compounds are also useful in anti-thrombotic therapy for pulmonary hypertension.

Therapeutic compounds according to this invention are preferably formulated in pharmaceutical compositions containing the compound and a pharmaceutically acceptable carrier. The pharmaceutical composition may contain other components so long as the other components do not reduce the effectiveness of the compound according to this invention so much that the therapy is negated. Some other components may have independent therapeutic effects. Pharmaceutically acceptable carriers are well known, and one skilled in the pharmaceutical art can easily select carriers suitable for particular routes of administration (*see, e.g., Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985). The concentrations of the active agent in pharmaceutically acceptable carriers may range from 0.01  $\mu\text{g/ml}$  to 500  $\mu\text{g/ml}$ .

The pharmaceutical compositions containing any of the compounds of this invention may be administered by parenteral (subcutaneously, intramuscularly, intravenously, intraperitoneally, intrapleurally, intravesicularly or intrathecally), topical, oral, rectal, or nasal route, as necessitated by choice of drug and disease.

Dose and duration of therapy will depend on a variety of factors, including the therapeutic index of the drugs, disease type, patient age, patient weight, and tolerance of toxicity. Dose will generally be chosen to achieve serum concentrations from about 0.1  $\mu\text{g/ml}$  to about 100  $\mu\text{g/ml}$ . Preferably, initial dose levels will be selected based on their ability to achieve ambient

- 18 -

concentrations shown to be effective in *in-vitro* models, such as that used to determine therapeutic index, and *in-vivo* models and in clinical trials, up to maximum tolerated levels. Standard clinical procedure prefers that chemotherapy be tailored to the individual patient and the systemic concentration of the chemotherapeutic agent be monitored regularly. The dose of a particular drug and duration of therapy for a particular patient can be determined by the skilled clinician using standard pharmacological approaches in view of the above factors. The response to treatment may be monitored by analysis of blood or body fluid levels of the compound according to this invention, measurement of activity of the compound or its levels in relevant tissues or monitoring disease state in the patient. The skilled clinician will adjust the dose and duration of therapy based on the response to treatment revealed by these measurements.

These compounds may also be applied locally or topically in gels, ointments, solutions, impregnated bandages, liposomes, or biodegradable microcapsules. Compositions or dosage forms for topical application may include solutions, lotions, ointments, creams, gels, suppositories, sprays, aerosols, suspensions, dusting powder, impregnated bandages and dressings, liposomes, biodegradable polymers, and artificial skin. Typical pharmaceutical carriers which make up the foregoing compositions include alginates, carboxymethylcellulose, methylcellulose, agarose, pectins, gelatins, collagen, vegetable oils, mineral oils, stearic acid, stearyl alcohol, petrolatum, polyethylene glycol, polysorbate, polylactate, polyglycolate, polyanhydrides, phospholipids, polyvinylpyrrolidone, and the like. For example, these compounds may be used to impede restenosis by application in a hydrogel at the time of angioplasty. A preferred strategy is to administer these compounds angioscopically in a suitable formulation.

A particularly preferred formulation for compounds according to this invention is in

- 19 -

liposomes. Liposomes containing compounds according to this invention may be prepared by any of the methods known in the art for preparation of liposomes containing inclusions. Liposomes that are particularly suited for aerosol application to the lungs are described in International Patent Publication WO 93/12756, pages 25-29, incorporated herein by reference.

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### **EXAMPLES**

In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

#### **10 Example 1. Production of vWF fragments with increased affinity for platelets.**

The vWF that contained the most frequent type 2b von Willebrand disease mutation -- a substitution of arginine by cysteine at aa residue 545 (R545C) -- has been expressed recombinantly (Inbal, et al., 1993, *Thromb. Haemost.*, 70:1058-1062). The recombinant R545CvWF protein exhibited an increased affinity for platelet GPIb. In order to study whether the increased binding of R545CvWF to platelets will remain within the truncated mutant vWF molecule after deletion of all the cDNA sequences except the putative GPIb binding domain, a recombinant fragment of vWF spanning from Ala444 to Asp730 containing the Arg545Cys mutation was constructed (Ala<sup>444</sup>-Asp<sup>730</sup>, named AR545CvWF).

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Using PCR-directed mutagenesis (Shymala, et al., 1991, *Gene*, 97:1-6), a truncated vWF cDNA fragment was designed that contained the native coding sequence for aa 444-730 and an arginine-by-cysteine substitution at aa 545. This mutated, truncated plasmid, denoted pSVAR545C was constructed from the full length R545CvWF plasmid by deleting sequences before Ala444 and

- 20 -

introducing the stop codon after Asp730. Two oligonucleotides that added an EcoR I restriction site at the 5' end of codon Ala 444 and at the 3' end of codon Asp 730 were employed for polymerase chain reaction (Shymala, et al., 1991, *Gene*, 97:1-6). The amplified fragment underwent EcoRI digestion followed by cloning within the EcoRI digested expression vector pZEM229. Thus, the resultant mutated plasmid, pSVAR545C, contained a fragment of vWF which comprises domains D3, A1 and part of A2 and the Arg545Cys mutation within the A1 domain. The sequence of each construct was verified by direct sequencing as described previously (Inbal, et al., 1993, *Thromb. Haemost.*, 70:1058-1062).

The recombinant wild type (pSUHvWF1) and mutated plasmid (pSVAR545C) were transfected in COS-7 cells by DEAE-dextran method as previously described (Inbal, et al., 1993, *Thromb. Haemost.*, 70:1058-1062; Englander, et al., 1996, *Blood*, 87:2788-2794). The recombinant proteins, wild type and AR545CvWF, were concentrated by ultrafiltration (Centriprep-30, Centricon-30, Amicon, Inc.) and quantitated by a sandwich ELISA using 1:1000 rabbit antihuman vWF (Dakopatts A082) as the coating antibody and 1:5000 peroxidase-conjugated anti-vWF antibody (Dakopatts P226) as the detecting antibody (Inbal, et al., 1993, *Thromb. Haemost.*, 70:1058-1062; Inbal, et al., 1993, *Blood*, 82:830-836). Western blot analysis of the recombinant AR545CvWF fragment using a monoclonal antibody that recognizes the A2 domain (36C4) disclosed a 116 kDa dimer that generated 52-48 kDa monomers upon reduction.

#### **Example 2. Inhibition of ristocetin-induced platelet aggregation by vWF fragments**

GP1b binding to wild type or AR545CvWF was analyzed in the absence or presence of ristocetin as described by Inbal, et al. (1993, *Thromb. Haemost.*, 70:1058-1062). In this method,

- 21 -

various concentrations of either recombinant wild type vWF or AR545wvWF fragments are incubated with formalin-fixed platelets ( $5 \times 10^8/\text{ml}$ ) for 15 min in a platelet aggregometer prior to the addition of 1  $\mu\text{g}/\text{ml}$  vWF and 1  $\text{mg}/\text{ml}$  ristocetin.

One microgram/ml of wild type or AR545CvWF was incubated with formalin-fixed platelets in the absence and presence of increasing concentrations of ristocetin. After 30 minutes of incubation, the bound platelet AR545CvWF complex was separated by centrifugation and the unbound vWF was quantitated by ELISA. In the absence of ristocetin, 60% of the AR545CvWF bound to platelets (spontaneous binding) and the binding increased to 80% at a ristocetin concentration of 1  $\text{mg}/\text{ml}$ . This increase contrasts with that of either absent or very little binding of wild type to platelets in the absence or at low concentrations of ristocetin, respectively.

### **Example 3. Large scale production and purification of the AR545CvWF fragment**

The recombinant vWF plasmid pSVAR545C was expressed in mammalian cells. The reason for choosing the mammalian transfection system is based on the fact that aa residues 445-507 that flank the A1 domain are glycosylated and the nonglycosylated *E. coli* derived material presents a neoantigenic site (Mohri, et al., 1993, *Peptides*, 14:125-129). Since the yield of COS-7 cell transfection was insufficient for the large scale production of the recombinant mutant vWF fragments, BHK cells were used.

### **Cell culture and expression of pSVAR545C**

A thymidine kinase-deficient BHK cell line, BHK-570, was used as the host cell for the transfection experiments (Meijers, et al., 1992, *Blood*, 79:1435-1440). Cells were grown in

- 22 -

Dulbecco's modified Eagle medium (DMEM), 5% fetal calf serum (FCS), 50  $\mu\text{g/mL}$  penicillin, 50  $\mu\text{g/mL}$  streptomycin, and 100  $\mu\text{g/mL}$  neomycin (Beit Ha'emek, Israel) in a 5%  $\text{CO}_2$  atmosphere at 37°C. For transfection, BHK-570 cells were plated at 1:15 split ratios in 90-mm plates (Falcon, Oxnard, CA) overnight and transfected for 4 hours in 10 mL of medium with 30  $\mu\text{g}$  of plasmid precipitated with calcium phosphate. After a 1-minute shock in 15% glycerol in Tris-phosphate-buffered saline (25 mmol/L Tris-HCl, pH 7.4, 0.14 mol/L NaCl, 5 mmol/L KCl, 0.7 mmol/L  $\text{CaCl}_2$ , 0.5 mmol/L  $\text{MgCl}_2$  and 0.6 mmol/L  $\text{Na}_2\text{HPO}_4$ ), the cells were grown for 24 hours in normal medium. The cells were then subjected to selective medium containing 1  $\mu\text{mol/L}$  methotrexate (Abic, Israel). The methotrexate concentration was increased gradually to 20  $\mu\text{mol/L}$ , and the clones were picked and propagated. At confluence, the cells were washed twice in PBS and cultured in 8 ml of serum free Dulbecco's modified Eagle medium/per T-75 culture flask. Medium was collected after 24 hours, and EDTA, phenylmethylsulfonyl fluoride, leupeptin and pepstatin were added to final concentration of 100  $\mu\text{M}$ , 100  $\mu\text{g/mL}$ , 1  $\mu\text{g/mL}$ , 1  $\mu\text{g/mL}$ , respectively. Conditioned media was concentrated by ultrafiltration (Amicor Inc. Beverly, CA, USA). Since the heparin binding site remained within the AR545C vWF fragment, for *in vivo* experiments the media was further purified on heparin affinity chromatography support (Econo-Pac® heparin cartridge, Bio Rad USA). The purity of the vWF fragment was verified by gel electrophoresis. The amount of vWF in conditioned medium was quantified by a sandwich ELISA using 1:100 rabbit anti-human vWF (Dakopatts A082) as the coating antibody and 1:1000 peroxidase-conjugated anti-vWF antibody (Dakopatts P226) as the detecting antibody. ELISAs were developed with o-phenylenediamine as the colorimetric substrate and quantitated at  $A_{490}$  on an ELISA reader (Molecular Devices, USA). The amino-terminal amino acid sequence of the AR545C vWF fragment was determined as a proof of identity



- 23 -

using standard procedures. The first 18 aa are: A E E A S G K K V T L N P S D P E H.

#### Ristocetin-induced platelet agglutination

Ristocetin-induced platelet agglutination was performed using lyophilized formalin-fixed platelets (Bio Data, Hartboro, PA) as described previously with slight modifications (Gralnick, et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89:7880-7884). Various concentrations of AR545C vWF were incubated with the platelets ( $2 \times 10^8$  platelets/ml) for 15 min. in a platelet aggregometer PACKS-4, (Helena Laboratories, Beaumont, Texas) at 37°C prior to the addition of 25  $\mu$ l of platelet poor plasma (PPP) as a source of vWF and 1 mg/ml ristocetin (Sigma, St. Louis, MO), and the percent agglutination was recorded. AR545C vWF inhibited ristocetin-induced platelet agglutination in a dose dependent manner with  $IC_{50}$  of  $0.80 \pm 0.04 \mu\text{mol/L}$ . The agglutination was completely abolished at 3  $\mu\text{mol/L}$  of AR545C vWF.

#### **Example 4. Anti-thrombotic properties of AR545CvWF**

Anti-thrombotic properties of the AR545CvWF fragment were analyzed using the rabbit femoral thrombosis model of Gold, et al. (1991, *Circulation*, 83:IV26-IV40). Briefly, a rabbit femoral artery was exposed and the vascular clamps were placed before and after the site of insertion of the superior epigastric artery (SEA). A cannula was inserted through SEA to induce thrombus. Distally the femoral artery was ligated to decrease the flow (measured by flow meter every 10 minutes) by 50%. The blood within the clamped segment was removed, the segment was traumatized by three compressions with blunt forceps to produce endothelial injury, and 100 $\mu$ l of rabbit atrial whole blood plus 40 units of bovine thrombin were injected to generate thrombus. Fifteen minutes after thrombus formation, both clamps were withdrawn and no flow was recorded.

- 24 -

To evaluate the anti-thrombotic properties of AR545CvWF, one hundred microliters of AR545CvWF fragment (1 $\mu$ g/ml) or mock transfected material (control) was injected through a cannula inserted within the superior epigastric artery, followed by a 450 $\mu$ g/kg bolus of tissue-type plasminogen activator (t-PA) as previously described (Gold, et al., 1991). The following parameters were measured: 1) time to thrombus formation; 2) time to first reperfusion; 3) time to reocclusion; 4) rate of reperfusion; and 5) total patency time. Eighteen New Zealand white rabbits were included in the study. Nine received the mutated fragment (AR545CvWF) and nine served as control, receiving mock transfected material. The results of these studies showed that the time to first reperfusion was significantly shorter in the experimental group than in the control (60.6 $\pm$  17.3 minutes vs. 103.0  $\pm$  15.2 minutes, respectively,  $p=0.05$ ). In addition, total patency time was also significantly increased in the group that received the mutated fragment compared with controls (175 minutes vs. 21 minutes,  $p=0.04$ ). With this protocol, however, there was no significant difference in either the time to first reocclusion ( $p=0.62$ ) or the reperfusion rate ( $p=0.15$ ), suggesting further benefit may be obtained by additional injections of the fragment or continuous IV infusion after the bolus injection.

#### **Example 5. Effect of Nitrosation on AR545CvWF**

S-NO-AR545CvWF has been synthesized and examined for its antiplatelet effects. S-NO-AR545CvWF was prepared by incubating AR545CvWF with a 3-fold excess of S-nitroso-glutathione in phosphate-buffered saline or with a 1.3-fold excess of NaNO<sub>2</sub> in 1 mM HCl for 15 minutes at room temperature. Excess S-nitroso-glutathione or NaNO<sub>2</sub> was removed by molecular sieve chromatography using a Sephadex G-25 column. Polynitrosation was performed as described

- 25 -

by Marks, et al. (1995, *Journal of Clinical Investigation*, 96:2630-2638); this procedure leads to incorporation of several S-NO groups per vWF fragment, and the resultant polynitrosated molecule is denoted pS-NO-AR545CvWF. The stoichiometry of S-nitrosation is determined using the Saville reaction (Saville, 1958. *Analyst*, 83:670-672).

#### Inhibition of Platelet Adhesion by AR545CvWF or S-NO-AR545CvWF

Platelet adhesion was assessed by determining the binding of platelets to endothelial cell monolayers or to extracellular matrix. Platelets were prepared from whole blood anticoagulated with 13 mM trisodium citrate and labeled with [<sup>111</sup>In]oxine. Endothelial cell monolayers or extracellular matrix surfaces were washed twice with Tyrode's solution at 37 C prior to the addition of 1 ml of labeled platelets (a total of  $5 \times 10^8$  platelets) to each well. An agonist used to induce the release of EDRF from the endothelial cell monolayers (e.g., bradykinin, substance P or acetylcholine), or AR545CvWF or S-NO-AR545CvWF were added 1 minute prior to the addition of platelets. In the case of extracellular matrix surface, platelets were preincubated with an NO-containing solution of AR545CvWF or S-NO-AR545CvWF for 1 minute prior to the addition of platelets. The platelets were incubated with the monolayers of matrix for 10 minutes at 37 C, after which the incubation suspension was removed, and the endothelial cells or matrix washed twice with Tyrode's solution at 37 C. The radioactivity remaining associated with the monolayers or matrix is indicative of adherent platelets, and it was liberated with 0.5% Triton X-100 and quantified in a  $\gamma$ -counter. The effect of nitrosated fragments of vWF can be measured using the procedure of Example 2.

The nitrosated fragment S-NO-AR545CvWF completely inhibited ristocetin-induced platelet agglutination at 0.8  $\mu$ M while the non-nitrosated fragment caused only 50% inhibition at the same

- 26 -

concentration. S-NO-AR545CvWF inhibited ristocetin-dependent platelet agglutination and also inhibited platelet adhesion in a flow chamber, and it did so 2.5-fold more potently than did the parent compound that was not nitrosated.

#### 5 Platelet aggregation using ADP and Thrombin

Importantly, S-NO-AR545CvWF also inhibits platelet aggregation in response to flow or in response to collagen or ADP, a property not shared by AR545CvWF. In some of the experiments the formalin-fixed platelets and purified vWF were substituted with platelet-rich plasma, and inhibition of ristocetin-induced aggregation was recorded by aggregometry. Platelet-rich plasma was diluted with platelet-poor plasma to give a final concentration of  $1.0 \times 10^5$  platelets/ $\mu$ l. Platelet aggregation was induced with 0.05 mM ADP or with thrombin (2.5 units/ml) plus L-glycyl-L-prolyl-L-arginyl-L-proline (1.5 mM final concentration) to prevent fibrin polymerization (Loscalzo, et al., 1986). Aggregation was monitored at 37 C while stirring (900 rpm) using a dualchannel aggregometer (Payton Associates, Inc., Buffalo). Aggregation was quantified by measuring the maximal rate of change in light transmittance.

The nitrosated fragment abolished ADP-induced platelet aggregation at 2.5  $\mu$ M, while the same concentration of non-nitrosated fragment still resulted in 60% aggregation.

#### Platelet interaction with extracellular matrix (ECM) in the cone and plate device (CAP)

20 Platelet adhesion and aggregation on ECM was tested. Briefly, 0.25 ml of citrated whole blood was placed on an ECM covered plate and subjected to arterial flow conditions (shear rate of  $1300 \text{ sec}^{-1}$ ) for 2 minutes by applying a cone and plate device, specially designed for this test. The

- 27 -

sample was then washed and stained with May Grunwald Stain (stain for peripheral blood cells including platelets) and the degree of adhesion (expressed as percent of surface coverage - SC) and aggregation (expressed as average size of the objects - AS) was determined using an image analysis system (Galai, Beit Ha'emek, Israel). Blood samples were preincubated at room temperature for 50 minutes with various concentrations of the nitrosated or non-nitrosated AR545C vWF and the extent of adhesion and aggregation was recorded. Using the CAP device, 0.2  $\mu$ M of the nitrosated fragment completely inhibited platelet aggregation and decreased the adhesion by 62%.

The nitrosated fragment exhibits significant antiplatelet properties, as demonstrated by a variety of tests for platelet adhesion and aggregation.

For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in medicine, pharmacology, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

- 28 -

CLAIMS:

1. A polypeptide having the following properties:

- (a) binds to platelet receptor glycoprotein Ib/IX (GPIb/IX);
- (b) competes with von Willebrand's Factor (vWF) for binding to GPIb/IX;
- 5 (c) does not activate platelets upon binding to GPIb/IX; and
- (d) contains at least one cysteine residue which is S-nitrosated.

2. The polypeptide according to claim 1, wherein the polypeptide contains at least a portion of the sequenced of the A1 domain of vWF.

10 3. The polypeptide according to claim 1, wherein said polypeptide is polythiolated and a plurality of thiol groups are nitrosated.

4. A substantially pure polypeptide containing at least the amino acid sequence of von Willebrand's factor (vWF) from ala at position 444 to asp at position 730, wherein the amino acid residue at position 545 is cys.

15 5. The polypeptide according to claim 4, said polypeptide consisting of the amino acid sequence of von Willebrand's factor from ala at position 444 to asp at position 730, wherein the amino acid residue at position 545 is cys.

6. The polypeptide according to claim 4, wherein at least one cys of said polypeptide is S-nitrosated.

20 7. The polypeptide according to claim 4, wherein said polypeptide is polythiolated and a plurality of thiol groups are nitrosated.

8. A method of treating a patient having an atherothrombotic disorder comprising administering a pharmaceutical composition containing the peptide according to claim 1 to the

- 29 -

patient.

9. A method of treating a patient having an atherothrombotic disorder comprising administering a pharmaceutical composition containing the peptide according to claim 3 to the patient.

5 10. A method of treating a patient having an atherothrombotic disorder comprising administering a pharmaceutical composition containing the peptide according to claim 4 to the patient.

10 11. A method of treating a patient having an atherothrombotic disorder comprising administering a pharmaceutical composition containing the peptide according to claim 6 to the patient.

12. A method of treating a patient having an atherothrombotic disorder comprising administering a pharmaceutical composition containing the peptide according to claim 7 to the patient.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/06092

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 17/00; A61K 38/00, 38/16

US CL :514/2, 8, 12; 530/324, 345, 380, 383, 395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 8, 12; 530/324, 345, 380, 383, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| Y         | US 5,321,127 A (HANDIN) 14 June 1994, column 3, lines 5 to column 4, lines 38.  | 1-2, 4-5, 8 and 10    |
| Y         | US 5,593,876 A (STAMLER et al) 14 January 1997, abstract.   | 1, 3, 6-9 and 11-12   |
| Y         | MARKS et al. Inhibition of Neointimal Proliferation in Rabbits after Vascular Injury by a Single Treatment with a Protein Adduct of Nitric Oxide. J. Clin. Invest. December 1995, Vol. 96, pages 2630-2638, especially page 2630. | 1, 3, 6-9 and 11-12.  |
| Y         | JACKSON et al. Adhesion Receptor Activation of Phosphatidylinositol 3-Kinase. The Journal of Biological Chemistry. 28 October 1994, Vol. 269, No. 43, pages 27093-27099, especially page 27093.                                   | 1-2                   |

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

|   |  |
|---|--|
| * Special categories of cited documents:  | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| *A* document defining the general state of the art which is not considered to be of particular relevance  | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| *B* earlier document published on or after the international filing date  | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
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| *O* document referring to an oral disclosure, use, exhibition or other means  |  |
| *P* document published prior to the international filing date but later than the priority date claimed  |  |

Date of the actual completion of the international search

14 MAY 1998

Date of mailing of the international search report

30 JUL 1998

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/06092

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| Y         | MATSUSHITA et al. Identification of Amino Acid Residues Essential for von Willebrand Factor Binding to Platelet Glycoprotein Ib. The Journal of Biological Chemistry. 02 June 1995, Vol. 270, No. 22, pages 13406-13414, especially page 13406. | 4-5                   |
| A,P       | LANKHOF et al. Functional Studies on Platelet Adhesion With Recombinant von Willebrand Factor Type 2B Mutants R543Q and R543W Under Conditions of Flow. Blood. 15 April 1997, Vol. 89, No. 8, pages 2766-2772.                                  | 1-7                   |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/06092

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, BIOSIS, WPIDS

search terms: platelet receptor glycoprotein, von Willebrand factor, glycoprotein Ib/IX or GPIb/IX, S-nitrosated, polythiolated, atherothrombic disorder or disease, treatment, pharmaceutical composition.